Gene silencing: **Two faces of SIR2**

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There are still many mysteries surrounding how silenced regions of the eukaryotic genome are created and maintained. But recent discoveries about the most evolutionarily conserved silencing protein, Sir2p, have provided new mechanistic insights into these processes.

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The great *Drosophila* geneticist, H.J. Muller, defined an 'ever-sporting' phenotype upon observing flies that had mosaic patterns of color in their eyes [1]. The phenotype arose after chromosome rearrangements were induced by X-ray irradiation that caused an eye color gene to be moved into centromeric heterochromatin. These experiments heralded the beginning of a genetic analysis of chromosome structure and its role in gene regulation. This phenomenon, later called position effect variegation, allowed biologists to appreciate that the structure of a particular region of the genome, as defined by its chromatin, confers special properties that can prevent gene expression, inhibit DNA recombination and replication, and affect chromosome segregation [2].

Specialized domains of chromatin exist throughout genomes of eukaryotes and are not limited to heterochromatin or centromeres. They extend to developmentally regulated regions of the genome, such as the homeotic gene cluster in *Drosophila*, to whole chromosomes, such as the inactive X chromosome in female mammals, or to the nucleolus, the home of ribosomal RNA synthesis [3–5]. Perhaps one of the best-defined systems for studying special chromatin domains has been the budding yeast, *Saccharomyces cerevisiae* [6]. *HML* and *HMR*, the loci that contain information specifying yeast mating type, were the first examples of 'silent' chromatin. Genes within these loci, while competent for transcription, are repressed as a result of the special chromatin structure they are packaged in.

The DNA in all eukaryotes is packaged as chromatin in units called nucleosomes, which consist of approximately 146 base pairs of DNA wrapped around an octamer consisting of two copies each of the histone proteins H2A, H2B, H3 and H4 [7]. The amino termini of each of the histones are tails that stick out of the nucleosome and are rich in lysines [8]. These amino-terminal tail lysines serve as sites of reversible covalent modifications, such as acetylation. In yeast silent chromatin, which includes telomeric regions of the genome as well as the silent mating-type loci, the amino-terminal tails of histone H3 and H4 are hypoacetylated relative to the rest of the genome [9]. Interestingly, hypoacetylation of histones H3 and H4 is also seen in silent chromatin of other organisms, such as in centromeric heterochromatin and along the inactive X chromosome in female mammals. These observations suggest that an understanding of how histones within silent chromatin become hypoacetylated is critical for elucidating the mechanisms of silent chromatin formation. Does hypoacetylation occur *in situ*, or are a special subset of hypoacetylated histones deposited specifically to silenced domains?

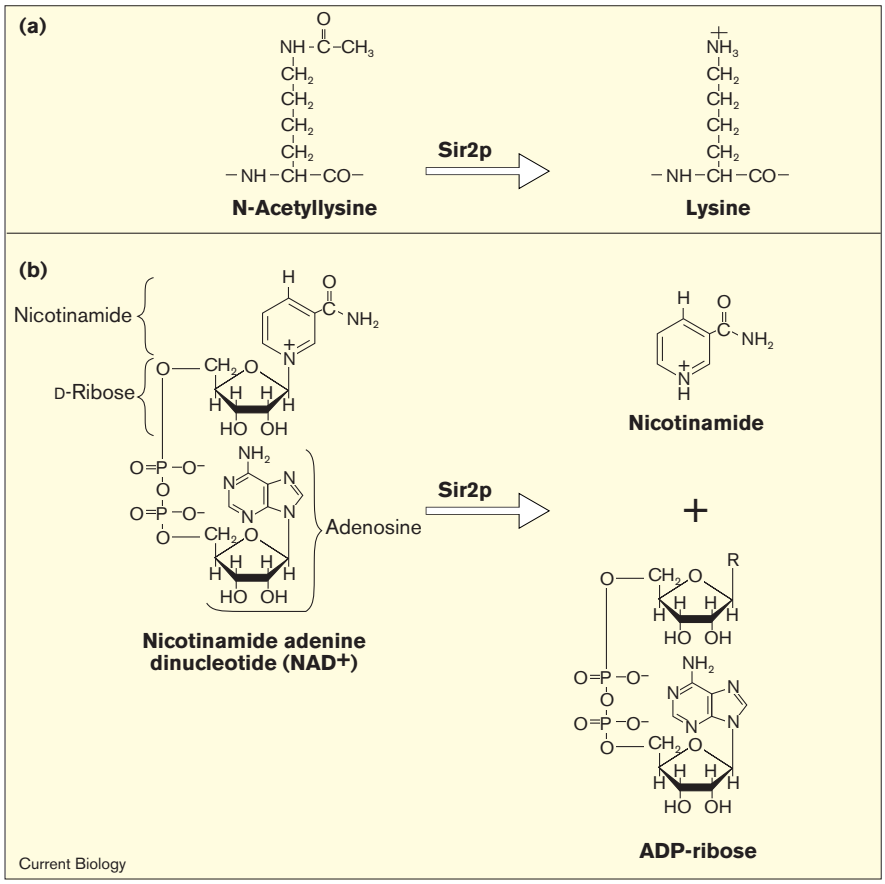
While the hypoacetylated histones H3 and H4 are the foundation for silent chromatin in yeast, other proteins are required as well [10]. The critical components are the 'silent information regulator' (Sir) proteins, Sir2p, Sir3p and Sir4p. Sir3p appears to blanket the underlying nucleosomes by interacting with the hypoacetylated histone H3 and H4 tails, thus impeding access to the DNA by transcription, replication and recombination machinery. Sir4p also interacts with the histone tails and may provide further stabilization of the silent chromatin structure. Sir3p and Sir4p are recruited to silent loci by interactions with sequence-specific DNA-binding proteins, such as Rap1.

Until recently, the role of Sir2p has been more enigmatic. While experiments indicate that Sir2p interacts with Sir3p and Sir4p in the silent chromatin complex, there was no compelling evidence that it binds to DNA or histones directly. Subsequent work from the laboratory of Jim Broach [11] hinted that Sir2p might be important in creating hypoacetylated histones. When Sir2p was highly overexpressed it was toxic to cells, but the histones within these cells appeared to be somewhat less acetylated than those in normal cells [11]. While there was no biochemical evidence that Sir2 was a deacetylase, this result proved to foreshadow Sir2p's function.

In addition to its roles in silencing the *HM* loci and genes located in telomeric regions of the genome, Sir2p is also involved in a special chromatin structure at the nucleolus in yeast, though it does so without the help of its Sir protein partners. In a complex with Net1p, Sir2p acts to inhibit recombination within the ribosomal (r)DNA array and reduce expression of the rDNA genes as well as RNA polymerase II-transcribed marker genes that are inserted within the array [12–14].

Figure 1

The two biochemical reactions carried out by Sir2 protein and its homologues. **(a)** Sir2p, acting as a deacetylase, removes an acetyl group from N-acetyllysine. **(b)** Sir2p catalyses the conversion of NAD^+ into nicotinamide and ADP-ribose. 'R' signifies the moiety that becomes ADP-ribosylated. It is unclear whether R is normally Sir2p or a different molecule. These two reactions appear to be interdependent; the deacetylase activity requires NAD^+, and the bond cleavage of NAD^+ is stimulated by acetyllysine.



Sir2p is the only Sir protein that is highly conserved [15]. In *S. cerevisiae* alone, there are four Sir2p homologues encoded by the *HST1–4* genes. Clear Sir2p homologues occur, not only throughout eukaryotes, but in eubacteria and archea as well. The significance of the similarity is unclear, however, as one of the homologues resides in the cytoplasm, and eubacteria have no histones nor are they known to have silent chromatin [16].

The discoveries

The first break in uncovering a biochemical activity from Sir2p came from work on one of its prokaryotic homologues, the S*almonella typhimurium* protein CobB [17]. CobB was found provisionally to substitute for CobT, an enzyme that catalyzes the transfer of 5′-ribose phosphate from nicotinate mononucleotide during the biosynthesis of vitamin B_{12}. Subsequent biochemical studies identified a nicotinamide adenine dinucleotide (NAD)-dependent ADP-ribosyltransferase activity associated with CobB and also with a human Sir2p homologue [18]. Shortly thereafter, a similar weak activity was reported for *S. cerevisiae* Sir2p, which was shown to transfer ADP-ribose from NAD to itself and to histones *in vitro* [19]. A point mutation in the conserved Sir2p domain that eliminated the *in vitro* ADP-ribose transfer activity also abolished silencing at loci normally affected *in vivo*. The mutant Sir2p could still interact with the other Sir proteins or Net1p, though its presence at silent loci was diminished. These observations suggested that Sir2p acts by ADP-ribosylating proteins, perhaps histones, at silent loci in order to create silent chromatin.

Following up on these observations, three papers [20–22] have been published this year that put a very interesting twist on the Sir2p enzymatic activity story. In one study [20], the analysis was based on principals learned, but often forgotten, in college biochemistry. Landry *et al.* [20] hypothesized that, if Sir2p has ADP-ribosyltransferase activity, then the protein might cleave the glycosidic bond between nicotinamide and ADP-ribose in NAD. This would create the following equilibrium: enzyme + NAD ↔ enzyme–ADP-ribose + nicotinamide. If the hypothesis were true, then incubating radioactive nicotinamide with Sir2p and unlabeled NAD would result in the NAD becoming radioactively labeled in an enzyme concentration- and time-dependent manner. No labeling of NAD occurred when Sir2p was the only protein in the mixture, but if histones isolated from chicken erythrocytes were added, then NAD became labeled.

Closer inspection of the histone requirement in the NAD exchange reaction revealed that the acetylated lysines in the amino-terminal tails were needed. In fact, replacement of acetylated histones with purified acetyl–lysine was sufficient to trigger the NAD exchange reaction. The NAD exchange reaction occurred at low levels of enzyme, but in contrast to the earlier studies, no transfer of ADP-ribose to histones was detected. When Landry *et al.* [20] turned their attention to the fate of the acetylated histones in these reactions, they made the exciting discovery that acetyl groups were removed from the lysines by Sir2p, or any of its homologues. Thus Sir2p appeared to be a deacetylase that uses NAD as a cofactor!

In a second study, Imai *et al.* [21] used chromatographic methods and mass spectroscopy to look for addition of an ADP-ribose moiety onto synthetic histone peptides [21]. While there was no evidence for such a modification with purified Sir2p and NAD, this group also discovered that when histone H3 or H4 peptides with acetylated lysines were used, the acetyl groups were removed during the incubation. Several previously characterized histone deacetylases are inhibited by drugs such as trichostatin A [23]. Incubation of Sir2p with this compound, however, had no effect on its deacetylase activity. This resistance to the drug contributes to the notion that Sir2p and its homologues represent a new class of deacetylase enzymes. These researchers also noted a weak ADP-ribosyltransferase activity when histone H3 peptides were a substrate. The weak activity could not, however, be detected by mass spectroscopy, only through the sensitivity of autoradiography.

In the third study, Smith *et al.* [22] confirmed that Sir2p and its homologues are NAD-dependent deacetylases, but they reported an additional interesting observation. In an earlier mutant hunt for genes required for rDNA silencing in yeast, a null allele of the *NPT1* gene was identified. This gene encodes a homologue of an enzyme involved in an NAD salvage pathway in bacteria. Consistent with the yeast protein Npt1p performing a similar function to its bacterial relative, levels of NAD were found to be reduced 2.5-fold in an *npt1*$^-$ strain. This provides *in vivo* support for the idea that Sir2p is an NAD-dependent enzyme. (This is a wonderful lesson for geneticists that think some of their non-obvious mutants are 'garbage'.)

Sir2 as a deacetylase.

The discovery that Sir2p is a lysine deacetylase (Figure 1a), which works on histones, provides a crucial missing piece in understanding how silent chromatin may be created and maintained. This activity offers a very plausible mechanism for the hypoacetylation of histones. It also raises the level of detail of the questions that can be asked. Does Sir2p deacetylate histones that are newly deposited upon the DNA during replication of silent chromatin domains? Such a scenario would permit its Sir partners to bind and blanket the nucleosomes. Is it required after DNA replication as well, to maintain silent chromatin? Though we may imagine structures such as silent chromatin as static, repressive walls, they are probably quite dynamic. Perhaps Sir2p is needed to maintain a proper foundation of hypoacetylated histones when Sir3 or Sir4p transiently leave the wall.

As a word of caution, a more important question to be answered is whether Sir2p's relevant silencing substrates are indeed histones. All the evidence says 'yes', but the smallest doubt must linger. *In vitro*, members of the Sir2 family of proteins are capable of working on all types of substrates — bovine serum albumin, for example — without significant specificity [18]. Most likely the proteins that Sir2 family members partner with determine the relevant substrates.

Sir2 and NAD

It is quite clear that the deacetylase activity of Sir2p requires NAD, and that this provides an interesting link between the metabolic energy flow of the cell and silencing. But the apparent activity of Sir2 and its homologues on NAD is distinct from our typical textbook knowledge of NAD. NAD is not used as a coenzyme by Sir2p to transfer electrons and H^+ ions, as it is in so many characterized reactions of the tricarboxylic acid cycle and other redox reactions. Instead, the evidence supports the idea that Sir2p cleaves the bond between nicotinamide and ribose-ADP (Figure 1b). The reason for this cleavage and the true fate of these two moieties remain to be determined.

While Sir2p and its homologues have ADP-ribosyltransferase activity *in vitro*, this activity seems to be quite weak compared to the deacetylase activity. Similarly, while Sir2p and its homologues, such as CobB, can also transfer nicotinamide to small molecule substrates when expressed in bacteria, it is unclear whether this is relevant to silencing. Perhaps energy gained by cleavage of the glycosidic bond is used to lower the activation energy of the deacetylase reaction, similar to how NAD is used as a cofactor by *Escherichia coli* DNA ligase [24]. Alternatively, NAD may be an allosteric effector.

Closing remarks

It is tempting to consider that Sir2p may serve as a node for connecting the networks of cellular physiology and genomic silencing. Perhaps when levels of NAD are reduced as a result of decreased precursor pools — such as those of tryptophan, glutamine and ATP — multiple genomic domains are 'opened' to explore alternative ways for bettering the cell's environment. Nevertheless, whatever roles Sir2p may play, there is still plenty of 'sport' left to understand chromatin domains of the genome.

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